Adenosine 3',5'-cyclic monophosphate-dependent release of prolactin from GH₃ pituitary tumour cells

A quantitative analysis

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The involvement of cyclic AMP in mediating regulatory peptide-controlled prolactin release from GH₃ pituitary tumour cells was investigated. Cholera toxin and forskolin elicited concentration-dependent increases in both GH₃ cell cyclic AMP content and prolactin release. The maximum rise in prolactin release with these agents was 2-fold over basal. 8-Bromo-cyclic AMP produced a similar stimulation of prolactin release. The phosphodiesterase inhibitor isobutylmethylxanthine also produced an increase in prolactin release and GH₃ cell cyclic AMP content. However, the magnitude of the stimulated prolactin release exceeded that obtained with any other agent. Thyrotropin-releasing hormone (thyroliiberin) and vasoactive intestinal polypeptide produced a concentration-dependent rise in both cell cyclic AMP content and prolactin release. However, only vasoactive intestinal polypeptide elicited an increase in cell cyclic AMP content at concentrations relevant to the stimulation of prolactin release. Vasoactive intestinal polypeptide and thyrotropin-releasing hormone, when used in combination, were additive with respect to prolactin release. Vasoactive intestinal polypeptide and forskolin, at concentrations that were maximal upon prolactin release, were, when used in combination, synergistic upon GH₃ cell cyclic AMP content but were not additive upon prolactin release. In conclusion the evidence supports a role for cyclic AMP in the mediation of vasoactive intestinal polypeptide- but not thyrotropin-releasing hormone-stimulated prolactin release from GH₃ cells. A quantitative analysis indicates that a 50–100% rise in cyclic AMP suffices to stimulate cyclic AMP-dependent prolactin release fully.

Prolactin release from anterior pituitary cells is known to be regulated by a variety of peptide hormones and releasing factors, although the mechanisms underlying this control remain elusive (for review, see Tixier-Vidal & Gourdji, 1981). Attempts to correlate biochemical changes with alterations in hormone release are complicated by the presence, in anterior-pituitary fragments or organ cultures, of a variety of cell types. GH₃ cells are a clonal strain of rat pituitary tumour cells that synthesize and secrete prolactin and growth hormone (somatotropin) (Tashjian *et al.*, 1968) and they provide a useful model system for investigating the mechanisms controlling prolactin release.

Abbreviations used: VIP, vasoactive intestinal polypeptide; TRH, thyrotropin-releasing hormone (thyroliiberin); IBMX, isobutylmethylxanthine.

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A number of studies have demonstrated that changes in the cellular content of cyclic AMP can influence prolactin release from GH₃ cells (see Tixier-Vidal & Gourdji, 1981). Agents such as cholera toxin and IBMX, which increase cell cyclic AMP levels, stimulate prolactin release and synthesis (Dannies *et al.*, 1976; Dannies & Tashjian, 1980). In addition, an increase in the GH₃ cell cyclic AMP content has been reported in response to the regulatory peptides VIP and TRH (Dannies *et al.*, 1976; Gourdji *et al.*, 1979; Drust *et al.*, 1982), although there is some controversy over this latter finding (Gershengorn, 1982). A possible complication in some of the experiments cited above is the fact that studies of prolactin release from GH₃ cells require that incubation times of less than 30 min be used. Measurements of released prolactin using longer incubation times can be influenced both by changes in release and in biosynthesis (Dannies & Tashjian, 1973).

In the present study we have investigated the role of cyclic AMP in the control of prolactin release by studying whether agents that modify prolactin release have effects on GH₃ cell cyclic AMP content and whether agents that act, by various mechanisms, to elevate cellular cyclic AMP levels influence prolactin release. The results indicate that a cyclic AMP-dependent pathway for prolactin release exists within GH₃ cells and that changes in the cell cyclic AMP content are tightly coupled to hormone release. Some of these results have already been presented in a preliminary form (Guild & Drummond, 1982).

Experimental

Culture of GH₃ cells

GH₃ cells are subclones of cells isolated from a rat pituitary tumour, the establishment and culture of which has been described previously (Tashjian *et al.*, 1968). The cells were grown in a humidified atmosphere of air/CO₂ (19:1) as a monolayer on plastic tissue culture dishes (93 mm diameter; Nunc-Gibco, Paisley, Scotland, U.K.) containing 10 ml of Ham's F-10 medium (Ham, 1963) supplemented with 15% (v/v) horse serum, 2.5% (v/v) foetal-calf serum, benzylpenicillin (100 i.u./ml) and streptomycin (100 µg/ml). They were plated [(5–10) × 10⁵ cells/dish] 7–10 days before use and were fed twice in the interim.

Measurement of GH₃ cell cyclic AMP content

On the day of the experiment cells were removed from the substrate using a rubber policeman and, together with those cells previously in suspension, were centrifuged out of the growth medium (200 g; 3 min; 25°C). The cell pellet was washed twice by resuspension/centrifugation in balanced salt solution {BSS; 135 mM-NaCl/4.5 mM-KCl/1.5 mM-CaCl₂/0.5 mM-MgCl₂/5.6 mM-glucose/10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/0.1% (w/v) bovine serum albumin, pH 7.4} to remove previously released prolactin. The cells were finally resuspended in BSS at a protein content of 0.1–0.2 mg/ml. Incubations were conducted in a volume of 1 ml. Drugs were added (10 µl) and the reaction carried out at 37°C in a shaking water bath. The incubations were terminated by placing the tubes into ice and by the addition of 4 vol. of ice-cold BSS. The cells were then centrifuged (200 g; 3 min; 4°C) and the supernatant retained for measurement of prolactin by radioimmunoassay. The cells were resuspended in 1 ml of 50 mM-Tris/HCl/4 mM-EDTA (pH 7.4) at 4°C and sonicated (10 W, 5 s) using a Dawe Soniprobe (Dawe Instruments, London W.3, U.K.). The sonicated cells were then placed in a boiling-water bath for

10 min to precipitate proteins. This protein was removed by centrifugation (1000 g; 15 min; 4°C) and the supernatant retained for the measurement of cyclic AMP levels. The protein content of the pellet was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V powder; Miles Laboratories, Slough, Bucks., U.K.) as standard. The cyclic AMP levels were measured by competitive protein-binding assay (Brown *et al.*, 1971).

Measurement of prolactin release from GH₃ cells

Immediately after the final cell suspension 1 ml portions of cell suspension were sampled for the determination of zero-time medium prolactin. This was subtracted from incubated values to give drug-accessible prolactin release. The measurement of rat prolactin levels in the samples was by radioimmunoassay using kit reagents kindly provided by Dr. A. Parlow, National Pituitary Agency, N.I.A.M.M.D.S., Bethesda, MD, U.S.A. The reference prolactin standard used was RP-2.

Hormones and chemicals

[8-³H]-Adenosine 3',5'-cyclic monophosphate (sp. radioactivity 962 GBq/mmol) was obtained from Amersham International, Amersham, Bucks., U.K. Na¹²⁵I (carrier-free) was from the Western Infirmary, Glasgow, Scotland, U.K., Culture materials were from Flow Laboratories, Irvine, Scotland, U.K., or Gibco-Europe, Paisley, Scotland, U.K. TRH and forskolin were from Calbiochem, Bishops Cleeve, Herts., U.K. VIP was a gift from Dr. V. Mutt, Karolinska Institutet, Stockholm, Sweden. Cholera toxin and all other chemicals were from Sigma, London S.W.6, U.K.

Results

The effects of thyrotropin-releasing hormone and vasoactive intestinal polypeptide

VIP produced a concentration-dependent increase in both prolactin release and GH₃ cell cyclic AMP content (Fig. 1). Threshold effects on prolactin release were observed at a concentration of 10 pM with a maximum effect (approx. 2-fold above basal release) around 10 nM. The EC₅₀ value (the concentration that produced 50% of the maximal response) was 200 pM. The dose-response curve for VIP-induced cyclic AMP accumulation was situated to the right of that for prolactin release: 75% of maximal VIP-induced prolactin release occurred with only a 25% increase in cell cyclic AMP content. VIP induced a 2-fold increase in cyclic AMP at 10 nM and higher concentrations were always associated with a reduced effect on cyclic AMP accumulation (Fig. 1; other results not shown). The

Fig. 1. *The effect of vasoactive intestinal polypeptide upon GH₃ cell cyclic AMP content and prolactin release* Cell suspensions (0.1–0.2 mg of cell protein/ml) were incubated for 5 min with VIP at the indicated concentrations. Cyclic AMP content (●) and prolactin release (▲) were measured as indicated in the Experimental section. Results are means $\pm$ S.E.M. of nine samples.

Fig. 2. *The effect of thyrotropin-releasing hormone upon GH₃ cell cyclic AMP content and prolactin release* Cell suspensions (0.1–0.2 mg of cell protein/ml) were incubated for 5 min with TRH at the indicated concentrations. Cyclic AMP content (●) and prolactin release (Δ) were measured as indicated in the Experimental section. Results are means $\pm$ S.E.M. of three samples.

EC₅₀ for VIP-induced cyclic AMP accumulation was 3 nM.

TRH also produced an increase in prolactin release and GH₃ cell cyclic AMP content (Fig. 2). Prolactin release was increased by TRH in the concentration range 10 pM–10 nM to a maximum of 2-fold above basal. Cyclic AMP, however, only accumulated in response to a much higher concentration of TRH (100 nM) (Fig. 2). Note that, in contrast with VIP, maximal TRH-induced prolactin release was not associated with an alteration in cell cyclic AMP content. If TRH and VIP stimulate prolactin via different mechanisms, it is possible that a combination of supramaximal concentrations of the two peptides will yield additive effects on prolactin release. The data in Fig. 3 confirm that this is, indeed, the case. In contrast, the VIP-induced elevation of cellular cyclic AMP content was unaffected by co-administration of TRH (10 nM).

The effects of forskolin, cholera toxin, isobutyl-methylxanthine and 8-bromo-cyclic AMP

Forskolin has previously been shown to elevate the cyclic AMP content in a variety of tissues (Seamon & Daly, 1981). The data in Fig. 4 indicate that forskolin could stimulate both prolactin release and cyclic AMP accumulation in GH₃ cells. As with both TRH and VIP, the maximum stimulated prolactin release was just over twice the basal rate. The EC₅₀ value for forskolin-induced prolactin release was 100 nM. It was not possible to investigate

Fig. 3. *The effect of vasoactive intestinal polypeptide and thyrotropin-releasing hormone when used alone, or in combination, upon GH₃ cell cyclic AMP content and prolactin release*

Cell suspensions (0.1–0.2 mg of cell protein/ml) were incubated for 5 min with the drugs as indicated. Cyclic AMP content (filled columns) and prolactin release (open columns) were measured as indicated in the Experimental section. Results are means $\pm$ S.E.M. of three samples.

the effects of forskolin at concentrations above 10 μ M in GH₃ cells because the vehicle, ethanol (0.4% v/v), caused a significant elevation of cyclic AMP levels on its own (results not shown). Treatment of GH₃

Fig. 4. The effect of forskolin upon GH_3 cell cyclic AMP content and prolactin release

Cells suspensions (0.1–0.2 mg of cell protein/ml) were incubated with forskolin at the indicated concentrations for 10 min. Cyclic AMP (●) and prolactin release (▲) were measured as indicated in the Experimental section. Results are means $\pm$ S.E.M. of nine samples.

cells with cholera toxin caused a maximal 30-fold accumulation of cyclic AMP after a discernible lag of 30 min (results not shown). Low concentrations of cholera toxin (1–10 μ M) elicited a 2–5-fold elevation of cell cyclic AMP content, which was associated with a stimulation of prolactin production (Table 1).

IBMX, a non-specific cyclic nucleotide phosphodiesterase inhibitor, also stimulated cyclic AMP accumulation in GH_3 cells (Table 1). Interestingly, a 3–4-fold elevation in cyclic AMP content resulting from the action of IBMX was associated with a far greater increase in prolactin release than that found with VIP or forskolin alone. Moreover, when a combination of forskolin and VIP was used to elevate cyclic AMP levels above those resulting from IBMX action, these markedly elevated levels were still unable to reproduce the stimulation of prolactin release found with IBMX alone (Table 1).

8-Bromo-cyclic AMP, a more lipophilic and phosphodiesterase-resistant analogue of cyclic AMP, which retains the ability to activate cellular cyclic AMP-dependent protein kinases (Revanker & Robins, 1982), stimulated prolactin release from GH_3 cells (Fig. 5) with an EC_{50} value of 4 μ M. The effects of 8-bromo-cyclic AMP on prolactin release were slower in onset than VIP, TRH or IBMX (results not shown), presumably reflecting the low

Table 1. The effects of drugs which stimulate cyclic AMP accumulation upon prolactin release from GH_3 cells. Cell suspensions (0.1–0.2 mg of cell protein/ml) were incubated at 37°C for 5 min with IBMX, VIP and forskolin and for 90 min with cholera toxin. Cyclic AMP and prolactin were measured as described in the Experimental section. Results are means $\pm$ S.E.M. for three samples.

Drug	Cyclic AMP (pmol/mg of protein)	Prolactin release (ng/mg of protein)
Controls	8.8 $\pm$ 0.3	60 $\pm$ 3
IBMX (1 mM)	33.5 $\pm$ 1.3	183 $\pm$ 3
VIP (10 nM)	15.4 $\pm$ 0.4	119 $\pm$ 4
Forskolin (1 μ M)	32.9 $\pm$ 1.5	123 $\pm$ 3
Forskolin (1 μ M) + VIP (10 nM)	160 $\pm$ 4.2	118 $\pm$ 5
Controls	8.4 $\pm$ 0.23	182 $\pm$ 8
Cholera toxin (1 μ M)	15.9 $\pm$ 1.5	453 $\pm$ 5
Cholera toxin (10 μ M)	43.1 $\pm$ 3	461 $\pm$ 11

Fig. 5. The effect of 8-bromo-cyclic AMP upon prolactin release from GH_3 cells

Cell suspensions (0.1–0.2 mg of cell protein/ml) were incubated for 20 min with 8-bromo-cyclic AMP at the indicated concentrations. Prolactin release was measured as indicated in the Experimental section. Results are means $\pm$ S.E.M. of three samples.

rate of penetration of 8-bromo-cyclic AMP to its intracellular site of action.

Fig. 6. A quantitative analysis of GH₃ cell cyclic AMP content against corresponding prolactin release

The cell cyclic AMP content and prolactin release data resulting from the forskolin and VIP studies reported in the present paper have been expressed as a percentage of mean control values and plotted to show the correlation between cyclic AMP content and prolactin release.

Quantitative aspects of cyclic AMP-induced prolactin release

In an effort to establish a quantitative relationship between cyclic AMP content and prolactin release, the data from a number of experiments involving VIP and forskolin were plotted as a percentage of control values (Fig. 6). It is evident that an increase in cyclic AMP content up to 150% of the basal value is coupled tightly to an increase in prolactin release. Further stimulation of cyclic AMP accumulation is redundant with respect to prolactin release.

Discussion

The objective of the present work was to determine whether alterations in cell cyclic AMP content could influence the release of prolactin from GH₃ cells, a clonal cell line used as a model system for the normal mammotroph. Two agents were studied that enhance adenylate cyclase activity. Cholera toxin has been reported to catalyse the ADP-ribosylation of the N_s-protein of the adenylate cyclase complex. As a result the toxin inhibits a GTPase associated with the N_s protein, which is the important off signal in the control of adenylate cyclase activity (Gill, 1977). This effectively leaves the catalytic unit of the adenylate cyclase complex locked in the activated position. In GH₃ cells, cholera toxin causes a marked enhancement of cyclic AMP production, after a lag time of 30 min (Dannies & Tashjian, 1980; Brostrom *et al.*, 1983,

other results not shown). Associated with this rise in cyclic AMP content is a stimulation of prolactin production. These data suggest that cyclic AMP is involved, although their interpretation in terms of prolactin release is complicated by the fact that long incubation periods are necessary to detect the action of cholera toxin. Prolactin production over these periods (in this case 90 min) represents the sum of changes in both release and synthesis. Such a problem does not occur with the second adenylate cyclase stimulant used. Forskolin, a diterpene of the labdane family, has been shown to enhance adenylate cyclase activity and cyclic AMP production in a number of tissues by a direct action on the catalytic subunit of adenylate cyclase (Seamon & Daly, 1981). The effects are rapid in onset and readily reversible. Recently Seamon & Daly (1983) have postulated that, in view of forskolin's specific action on adenylate cyclase, another criterion for the involvement of cyclic AMP in a physiological process would be that forskolin should stimulate the response.

In GH₃ cells forskolin caused a doubling in cyclic AMP content that was associated with enhanced prolactin release. In confirmation of previous work in other tissues, forskolin and VIP were synergistic with respect to cyclic AMP production. This is the basis of another report from this laboratory (Drummond & Guild, 1983). If cyclic AMP can mediate prolactin release from GH₃ cells induced by cholera toxin and forskolin then it or a more appropriate analogue of cyclic AMP, 8-bromo-cyclic AMP, was work has shown that dibutyryl cyclic AMP can stimulate prolactin release from GH₃ cells (Dannies *et al.*, 1976), although sodium butyrate itself has a similar effect (Dannies *et al.*, 1976; Yen & Tashjian, 1981). In the present study a metabolism-resistant analogue of cyclic AMP 8-bromo-cyclic AMP, was investigated and found to elicit prolactin release at relatively low concentrations. Lastly, in this section we investigated whether a phosphodiesterase inhibitor, IBMX, could influence prolactin release. This was shown to be the case, although the maximal degree of stimulation evoked by IBMX exceeded that elicited by forskolin, cholera toxin or 8-bromo-cyclic AMP. It seems likely that IBMX, in addition to stimulating cyclic AMP-dependent prolactin release, exerts a further effect unrelated to changes in cyclic AMP metabolism (see below).

A second objective of the study was to determine whether the cyclic AMP-dependent pathway for prolactin release is utilized by any of the regulatory peptides that influence prolactin release. These include agents such as VIP (Ruberg *et al.*, 1978), TRH (Tashjian *et al.*, 1971) and bombesin (Westendorf & Schonbrunn, 1982). There is evidence that both VIP and TRH may act through the aegis of cyclic AMP (for review, see Tixier-Vidal & Gourdji,

1981). VIP has been shown to enhance adenylate cyclase activity in GH₃ cell homogenates (Martin & Ronning, 1981; Brostrom *et al.*, 1982) and to increase the cyclic AMP content of the pituitary cells (Gourdji *et al.*, 1979; Drust *et al.*, 1982; the present work). Further evidence supporting the involvement of cyclic AMP in mediating VIP-stimulated prolactin release is the finding shown in the present paper that the effect of a supramaximal concentration of VIP is not additive with a similar concentration of forskolin and also the finding that there is a correlation observed between the VIP-elevated cyclic AMP content and prolactin release. The data in the present paper and previous reports satisfy the criteria of Sutherland for the involvement of cyclic AMP in mediating VIP-induced prolactin release. The data for TRH are far less convincing. Although one group has reported TRH-sensitive adenylate cyclase activity in GH₃ cell homogenates (Gautvik *et al.*, 1982), this has not been confirmed by others (Hinkle & Tashjian, 1977; A. H. Drummond & W. K. Pollock, unpublished work). Some studies indicate that TRH does not affect the GH₃ cell content of cyclic AMP (reviewed in Gershengorn, 1982). However, we and others (Dannies *et al.*, 1976; Gautvik *et al.*, 1980; Brostrom *et al.*, 1983) show a clear stimulatory effect especially at relatively high concentrations of TRH. The data suggest, however, that TRH concentrations that enhance prolactin release do not affect the cellular content of cyclic AMP.

These facts may be reconciled by the recent finding that TRH-induced cyclic AMP production in GH₃ cells is dependent on cellular Ca²⁺ (Brostrom *et al.*, 1983). It is conceivable that a TRH-induced rise in cytoplasmic free [Ca²⁺], as proposed by Gershengorn (1982), may subsequently mediate adenylate cyclase stimulation. This would explain the inability of most workers to detect TRH-stimulated adenylate cyclase activity in broken cell preparations of GH₃ cells. Further evidence in favour of the view that TRH stimulates prolactin release independent of cyclic AMP changes is the finding that maximally active concentrations of TRH and VIP are additive with respect to prolactin release and the results of phosphorylation studies carried out by Drust *et al.* (1982). These indicate that VIP and TRH stimulate the phosphorylation of distinct populations of proteins in GH₃ cells.

It is interesting that IBMX can enhance prolactin release to the same extent as a combination of VIP and TRH. Xanthines have been shown to influence cellular Ca²⁺ distribution in a number of tissues (Clusin, 1983) and IBMX has recently been shown to enhance phosphatidylinositol turnover in GH₃ cells in a manner similar to TRH (Sutton & Martin, 1982). It is possible that both the cyclic AMP-dependent pathway of prolactin release used by VIP

and the Ca²⁺-dependent pathway used by TRH are activated by IBMX.

Since GH₃ cells are derived from a pituitary tumour they are unlikely to represent exactly the normal lactotroph. Nevertheless the cells do respond in the conventional manner to most of the agonists that affect prolactin release in their non-transformed counterparts e.g. TRH, VIP and somatostatin (Tixier-Vidal & Gourdji, 1981). Two differences between GH₃ cells and normal lactotrophs are the virtual absence of dopamine receptors in GH₃ cells (for review see Gourdji *et al.*, 1982) and the relatively small amounts of prolactin stored by the tumour cells: treatment of GH₃ cells for 5 min with a saturating concentration of VIP or TRH causes a 30–40% depletion of the cellular stores of prolactin (S. Guild & A. H. Drummond, unpublished work). It seems unlikely, however, that the intracellular mechanisms that control prolactin release will be radically different in the two types of cells. A comparison of the data in the present paper with those from anterior pituitary fragments or dispersed cell cultures is difficult owing to the heterogeneity of cell types present in normal tissue. Nevertheless support for a role for cyclic AMP in the control of prolactin release from normal lactotrophs has come from studies using cholera toxin, IBMX and 8-bromo-cyclic AMP (Tam & Dannies, 1981). In addition, VIP has been shown to increase both anterior pituitary cyclic AMP levels and prolactin release (Ruberg *et al.*, 1978; Samson *et al.*, 1980).

In summary, these results demonstrate that cyclic AMP can control prolactin release from GH₃ cells but that only a 50–100% increase in the nucleotide content is necessary to activate this pathway maximally. Tight coupling of this type between cyclic AMP content and a cellular response have been reported now in a variety of situations (Brooker, 1975; Yamamura *et al.*, 1976; Drummond *et al.*, 1977) and must be taken into account in experiments designed to test the hypothesis that cyclic AMP controls a particular physiological response.

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